Lack of Toxicity to Adults of the Mexican Fruit Fly (Diptera: Tephritidae) of β-Exotoxin in *Bacillus thuringiensis* Endotoxin Preparations

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ABSTRACT   β-Exotoxin (thuringiensin) was found in high titers in centrifugation supernatants and acetone/lactose powders produced from centrifugation pellets of strains Guat 1 and HD 2 of *Bacillus thuringiensis* (Berliner). Diets containing powders of either strain were toxic, diets containing Guat 1 supernatant were not toxic, diets containing HD 2 supernatant were slightly toxic, and diets containing powders or supernatants from uninoculated culturing medium spiked with β-exotoxin were not toxic. Most mortality occurred within 3 d when flies fed on powders but not until 6–7 d when flies fed on HD 2 supernatant. These results indicated that the primary toxic principals of the powders were endotoxins/spores and that β-exotoxin alone was not toxic to adult flies at the concentrations found in the supernatants or powders.

KEY WORDS   *Anastrepha ludens, Bacillus thuringiensis*, β-exotoxin, thuringiensin, endotoxin, biological insecticides

ENDOTOXINS AND EXOTOXINS produced by *Bacillus thuringiensis* (Berliner) have been used as insecticides in various ways around the world during the last 40 yr (Sebesta et al. 1981, Starnes et al. 1993). The most-characterized *B. thuringiensis* exotoxin is β-exotoxin or thuringiensin discovered by McConnell and Richards (1959). This adenine nucleotide analog has a broad spectrum of pesticidal activity to larval insects (Krieg and Langenbruch 1981). However, β-exotoxin is not registered for use in the United States because it interferes with RNA synthesis in vertebrates as well as invertebrates and is deemed an environmental hazard by the Environmental Protection Agency (Mc-Clintock et al. 1995). Companies in the biopesticide industry that work with *B. thuringiensis* products routinely screen for this chemical and stop development of any product that contains it.

Endotoxin/spore preparations (containing the spore/crystal complex) including centrifugation pellets and acetone/lactose powders produced from the pellets have been shown to have insecticidal activity to adults of Mediterranean fruit flies, *Ceratitis capitata* (Wiedemann) (Gingrich 1987); olive fruit flies, *Bactrocera oleae* (Gmelin) (Bel et al. 1997), and Mexican fruit flies, *Anastrepha ludens* (Loew) (Robacker et al. 1996, Martinez et al. 1997). Gingrich (1987) hypothesized that the toxicity of his pellets/powders was partially caused by endotoxins/spores and partially caused by water-insoluble exotoxins, including β-exotoxin that may have precipitated as a calcium salt from the supernatant. Dulmage (1981) confirmed the presence of β-exotoxin in powders of some strains, giving credence to Gingrich's hypothesis. Robacker et al. (1996) and Martinez et al. (1997) credited the toxicity of their powders to endotoxins/spores for three reasons. First, powders autoclaved at pH 7 were no longer toxic to the flies. Autoclaving at pH 7 reportedly destroys protein endotoxins and spores (Gingrich 1987). Second, autoclaving at pH 7 should not affect β-exotoxin (Campbell et al. 1987, Levinson et al. 1990), indicating β-exotoxin was not the toxic principal. Third, we considered β-exotoxin to be only weakly toxic. Dulmage (1981) stated that the activity of β-exotoxin is much weaker than that of endotoxins and the presence of "β-exotoxin in a powder does not materially affect the interpretation of our data on the δ-endotoxin."

Because initial analyses of the powders used by Robacker et al. (1996) and Martinez et al. (1997) indicated that β-exotoxin was present, we investigate here if β-exotoxin is toxic to adult Mexican fruit flies in amounts in our powders. Concentrations of β-exotoxin were quantified by HPLC in supernatants and powders of Guat 1, our most toxic strain in previous work, and HD 2, a strain known to produce β-exotoxin (Levinson et al. 1990). Toxicities of diets containing powders and supernatants of Guat 1 and HD 2 were assessed along with diets containing β-exotoxin only. Possible roles of β-exotoxin and endotoxins/spores in toxicity of the preparations are discussed.

Materials and Methods

Fermentation and Preparation. The *Bacillus thuringiensis darmstadiensis* strain Guat 1 was obtained

from a soil sample from Guatemala and was described previously (Martinez et al. 1997). *B. t. thuringiensis* strain HD 2 was obtained from the Northern Regional Research Laboratory *B. thuringiensis* culture collection (USDA–ARS, Peoria, IL). Fermentation medium, fermentation procedures, harvesting procedures, and acetone/lactose powder (dried precipitate formed by the procedure) preparation were also described previously (Robacker et al. 1996).

Insects. Flies were from a laboratory colony at the ARS facility at Weslaco, TX. The colony originated from adults that emerged from yellow chapote fruit (*Sargentia greggii* S. Wats) collected at Montemorelos, Nuevo Leon, Mexico, in 1994. The culture had been maintained on artificial diet since that time. Adult flies were fed sugar and water until bioassays were conducted, usually about 1 wk after eclosion.

High-Performance Liquid Chromatography. β-Exotoxin concentrations were determined in centrifugation supernatants, rinses of centrifugation sediments, and acetone/lactose powders by high-performance liquid chromatography (HPLC). The HPLC was a Waters system including a model 717plus Autosampler, model 600E Multisolvent Delivery System with model 60 F Pump, and model 996 Photodiode Array Detector (Waters, Milford, MA). The system was controlled by a NEC Image 466es computer (NEC Technologies, Inc., Boxborough, MA) with Millenium 2010 Chromatography Manager Software. The HPLC column was a Waters μ-Bondapak C18 (3.9 mm by 30 cm, 125 A, 10-μm particle size). Mobile phase was 50 mM KH_2PO_4 buffer (pH 3) (Campbell et al. 1987) at 1 ml/min. UV spectra were recorded every 4 s throughout analyses from 215 to 295 nm (1.2-nm intervals).

Determination of β-Exotoxin in *B. thuringiensis* Preparations. β-Exotoxin standard labeled 'β-exotoxin Lot # 52-595-BD, 1983', originally from Abbott Laboratories (Long Grove, IL), was obtained from the laboratory of H. Dulmage (USDA–ARS, Brownsville, TX). Retention volumes and UV absorption spectra of the major peak in the standard were compared with peaks in HD 2 and Guat 1, and with those reported in literature for β-exotoxin (Campbell et al. 1987, Levinson et al. 1990). β-Exotoxin in HD 2 and Guat 1 was quantified with a calibration curve constructed using standard β-exotoxin.

β-Exotoxin in supernatants and rinses (four rinses of centrifugation sediments with water) was measured by direct injection of the aqueous samples. β-Exotoxin in powders was measured after extraction of the toxin from the insoluble powders by sonication for 30 min in 50 mM KH_2PO_4 buffer (pH 3). The purpose of the buffer was to solubilize β-exotoxin/calcium salts (Campbell et al. 1987) formed from calcium in the fermentation medium. The sonication procedure was repeated until the amount of β-exotoxin extracted from the samples was below detection level. Quantitations were conducted for four fermentations of Guat 1 and one of HD 2.

Diet Incorporation of *B. thuringiensis* Powders, Supernatants, and β-Exotoxin. Twelve adult-fly diets were prepared to determine the relative toxicities of Guat 1 powders containing spore/crystal complex and β-exotoxin, centrifugation supernatants containing β-exotoxin, and β-exotoxin alone. These diets were compared with other diets containing similar preparations of HD 2 and with control diets containing β-exotoxin or no bacterial components. All diets contained either an acetone/lactose powder (83%) or supernatant (83%) and sugar (17%).

The first four diets were made with acetone/lactose powders containing amounts of β-exotoxin determined by HPLC. The first was Guat 1 powder (combined powders of the four fermentations of Guat 1). The second diet was HD 2 powder. The third was a control diet containing powder prepared from uninoculated fermentation medium (medium B powder diet). The fourth diet was like the third except it was spiked with β-exotoxin standard to equal the concentration in the Guat 1 powder diet.

The other eight diets were prepared with concentrated centrifugation supernatants containing amounts of β-exotoxin determined by HPLC. Supernatants were concentrated to 5–10% of original volume in a rotary evaporator at pH 7 for incorporation into diets. The first supernatant diet was concentrated Guat 1 supernatant (combined concentrated supernatants of the four fermentations of Guat 1). The second supernatant diet was concentrated HD 2 supernatant. Two control diets were made with concentrated supernatant of uninoculated fermentation medium. One of these was spiked with β-exotoxin at the concentration of the Guat 1 supernatant diet. These four supernatant diets were prepared at pH 7. The last four supernatant diets were identical to the first four except they were prepared at pH 4 as a precaution against precipitation of the β-exotoxin with calcium present in the fermentation medium.

Diets were fed to flies in Plexiglas cages (20.3 cm per side) containing 21–25 flies for 10 d. Sugar cubes were removed from the cages when the diets were introduced then replaced after 2 d. Flies were observed to feed on all diets, although it was not certain that all flies fed on the diets. Additional diet was added as needed to replace diet consumed by flies. Laboratory conditions were $22 \pm 2°C$, $55 \pm 20\%$ RH, and a photoperiod of 13:11 (L:D) h. Ten replications were conducted, each replication including one cage for each of the 12 diets. Percentage mortality after 7 d was analyzed by two-way analysis of variance (ANOVA) separating effects of replication and diet. Analyses were conducted using procedure SuperANOVA (Abacus Concepts 1989).

Results and Discussion

Determination of β-Exotoxin in *B. thuringiensis* Preparations. β-Exotoxin was found in supernatants, rinses and powders of Guat 1. Centrifugation supernatant contained 97 ± 8.6 μg/ml (SEM) of β-exotoxin (n = 4 fermentations) for a total of 48 mg per fer-

Table 1. Mortality (mean percentage ± SEM) of adult Mexican fruit flies fed diets containing acetone/lactose powders or supernatants of two *B. thuringiensis* strains, uninoculated fermentation medium and uninoculated fermentation medium spiked with β-exotoxin

Diet	Concn of β-exotoxin (mg/g)	Mortality
Guat 1 powder	5.8	42.7 ± 5.5e
HD 2 powder	1.3	56.4 ± 4.5f
Medium B powder	0	13.2 ± 5.7d
Medium B powder + β-exotoxin	5.8	10.5 ± 5.5bcd
Guat 1 supernatant (pH 7)	1.0	4.1 ± 1.3ab
Guat 1 supernatant (pH 4)	1.0	4.3 ± 1.4abc
HD 2 supernatant (pH 7)	0.7	4.4 ± 1.1abc
HD 2 supernatant (pH 4)	0.7	13.0 ± 4.8cd
Medium B supernatant (pH 7)	0	1.3 ± 0.9a
Medium B supernatant (pH 4)	0	1.2 ± 0.9a
Medium B supernatant + β-exotoxin (pH 7)	1.0	2.5 ± 1.1ab
Medium B supernatant + β-exotoxin (pH 4)	1.0	1.2 ± 0.6a

Means followed by the same letter are not significantly different at ($P < 0.05$; Fisher protected least significant difference method [Fisher 1935]; $n = 10$ for each diet).

Fig. 1. Cumulative mortality (mean percentage ± SEM) as a function of time after Mexican fruit flies began feeding on diets containing *B. thuringiensis* preparations (SEM < 1.6% not shown).

mentation (one fermentation, 500 ml). Rinses of the centrifugation pellet with water contained 38 ± 1.0 µg/ml ($n = 4$) for a total of 19 mg from four rinses of 125 ml each. Acetone/lactose powder contained 6.9 ± 0.58 mg/g ($n = 4$) for a total of 81 mg per fermentation (11.8 g of powder produced per fermentation). Total β-exotoxin produced per fermentation (500 ml) was 148 mg.

The HD 2 fermentation that was used to prepare diets for bioassay had concentrations (mean of three determinations of one fermentation ± SEM) of 760 ± 37 µg/ml of β-exotoxin in the supernatant (500 ml) and 1.6 ± 0.070 mg/g in the powder (14.4 g). Total β-exotoxin could not be calculated because concentrations in rinses were not determined.

Although acetone/lactose powders are generally regarded as endotoxin preparations from which water-soluble chemicals have been removed by thorough water rinsing, our results clearly demonstrate they may contain water-soluble β-exotoxin (thuringiensin) under some conditions.

Toxicity of Diets Containing β-Exotoxin and Endotoxins/Spores. Diets containing powders of Guat 1 and HD 2 were significantly ($F = 32.7$; df = 11, 99; $P < 0.0001$) more toxic to flies than control diets containing powder from uninoculated culturing medium (medium B powder) (Table 1). This is the first report that acetone/lactose powders of HD 2 are toxic to adult fruit flies. Medium B powder diet spiked with β-exotoxin was not more toxic than medium B powder diet without β-exotoxin. Diets containing Guat 1 supernatant or HD 2 supernatant at pH 7 were not more toxic than control diets containing supernatant of medium B. Diets containing medium B supernatant spiked with β-exotoxin also were not toxic. The diet containing HD 2 supernatant at pH 4 was significantly more toxic than control diets with supernatant of medium B but was less toxic than the HD 2 and Guat 1
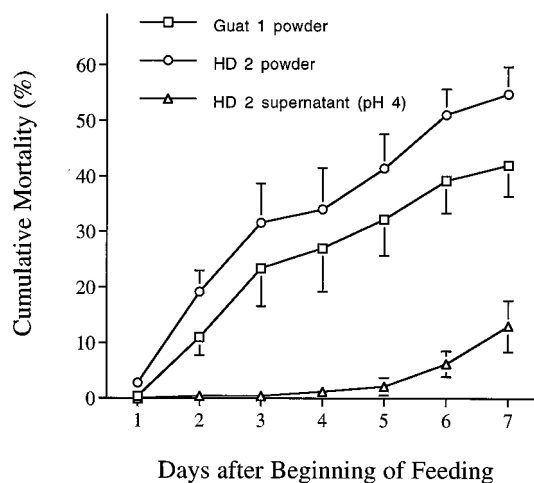
powder diets. Previously, we showed that aqueous formulations of Guat 1 powders were most toxic to Mexican fruit flies at pH 4 and were not toxic at pH 7 (Martinez et al. 1997).

Guat 1 and HD 2 powders not only killed more flies but also killed them faster than HD 2 supernatant (Fig. 1). More than half of the mortality occurred within 3 d after flies began feeding on the powders compared with 6 d after feeding on HD 2 supernatant. Further, almost no flies died within 3 d after feeding on HD 2 supernatant. These results suggest different modes of action of the killing agents in the two types of preparations. Moar et al. (1986) obtained similar results with *Spodoptera exigua* (Hübner) larvae fed diets containing either β-exotoxin or *B. thuringiensis* spore/crystal complex of HD 1, a strain that does not produce β-exotoxin. In their study, most mortality occurred by day 3 with the HD 1 spore/crystal complex and between days five and six with β-exotoxin.

HD 2 powder was more toxic than Guat 1 powder and HD 2 supernatant was more toxic than Guat 1 supernatant despite lower titers of β-exotoxin in the HD 2 preparations. The greater toxicity of HD 2 compared with Guat 1 suggests the possibility that yet another toxic agent is present in the HD 2 preparations. Evidence indicates the agent may be another type of exotoxin because it is present in supernatant and because the time course of its action was similar to that of β-exotoxin in the study of Moar et al. (1986).

Our results do not prove that β-exotoxin had no effect on toxicity in the Guat 1 and HD 2 powders. The results demonstrate only that β-exotoxin alone was not toxic to adult Mexican fruit flies in the concentrations present in either Guat 1 or HD 2 powders. We cannot rule out the possibility that the toxicity of the powders may have resulted from a synergistic effect of β-exotoxin with the endotoxins/spores. Such synergism (potentiation) has been reported with diets fed to *S.*

exigua larvae (Moar et al. 1986). In that case, however, β-exotoxin was significantly toxic alone, and the combination of β-exotoxin with the spore/crystal complex was more toxic than the additive effects of the two agents.

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